Reference: *Biol. Bull.* 173: 504–512. (December, 1987)

QUANTITATIVE ESTIMATION OF MOVEMENT OF AN AMINO ACID FROM HOST TO *CHLORELLA* SYMBIONTS IN GREEN HYDRA

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Abstract

Washing symbiotic *Chlorella* algae freshly isolated from green hydra with 0.05% sodium dodecyl sulphate was shown to remove virtually all contaminating host material, previously a severe constraint in quantifying movement of metabolites from host to symbionts. When brine shrimp labelled with ^{3}H-leucine were fed to hydra in symbiosis either with the native strain of *Chlorella* (E/E hydra) or two strains cultured from *Paramecium bursaria* (E/3N and E/NC hydra), it was found that after 24 h 3–4% of the total radioactivity retained by the symbiosis was present in the algae. Analysis of the free amino acid pool of symbiotic algae from E/E hydra showed that over 70% of the radioactivity was associated with leucine, and significant amounts of radioactivity were retained by these algae for at least five days following a single feeding with radioactive brine shrimp. In both E/E and E/NC hydra, the amount of radioactivity per unit protein was considerably less in the symbionts than in the host, suggesting that access to host amino acid pools were limited. These results are discussed in terms of the possible role and regulatory significance of amino acids as a nitrogen source to symbiotic *Chlorella*, and of the cost to the host in maintaining the symbiosis.

Introduction

While it is well known that *Chlorella* algae symbiotic with green hydra release photosynthetically fixed carbon that is used by their hosts (Cernichiari *et al.*, 1969; Mews, 1980; Mews and Smith, 1982), it has become apparent only recently that movement of metabolites in the opposite direction may also be important in maintaining the symbiosis. In hydra grown in darkness, host supply of metabolites, which from indirect evidence may include glucose (McAuley, 1986a), is necessary to maintain a reduced population of symbionts. In light, cell division of the algae appears to be dependent upon a 'factor' present in host food, possibly one or more amino acids (McAuley, 1985, 1986b).

Amino acids may also have an important role in nitrogen supply to the symbionts. Although it was long believed that algae symbiotic with green hydra used ammonium produced by host catabolic processes, as in symbioses between marine invertebrates and zooxanthellae (Muscatine, 1980; Wilkerson and Muscatine, 1984; Summons *et al.*, 1986; Anderson and Burris, 1987), recent research has shown that high levels of host glutamine synthetase and low perialgal vacuolar pH may prevent uptake of ammonium by algae in green hydra (Rees, 1986). Since the symbiotic algae also lack nitrate reductase and nitrite reductase (D. McKinney and H. Lenhoff, University of California, Irvine, pers. comm.), it has been suggested that host supply of amino acids

Received 17 July 1987; accepted 30 September 1987.

may form the primary source of nitrogen for algae symbiotic with green hydra (McAuley, 1986b, 1987; Rees, 1986).

Direct evidence for movement of metabolites from host to symbiotic algae has been difficult to obtain. Logically, it could be studied by supplying food containing radioactively labelled metabolites, then isolating algae from the symbiosis and assessing uptake of radioactivity. Although this approach has been used (Cook, 1972; Thorington and Margulis, 1981), the interpretation of the results is now in doubt because it was subsequently realized that algae isolated by centrifugation of homogenates of green hydra are heavily contaminated with host material, despite washing the algal pellet in several changes of distilled water or culture medium (Cook, 1983; Douglas and Smith, 1983; McAuley, 1986c).

Recently, it was demonstrated that complete removal of contaminating host material could be achieved by washing the algal pellet with a dilute solution (0.05%, w:v) of the surfactant sodium dodecyl sulphate (SDS) (McAuley, 1986c). Meints and Pardy (1980) earlier used SDS to isolate algae from green hydra, but at a higher concentration which was subsequently found to affect the viability of the algae. However, neither the viability, nor the photosynthetic capacity, nor the ability to sequester and retain amino acids were affected after washing with 0.05% SDS (McAuley, 1986c; Douglas, in press). In this paper the SDS-washing technique was used to separate algae from host material in homogenates of hydra which had been supplied with food labelled with ^{3}H-leucine. Evidence for the transfer of leucine from host food to the symbiotic algae is described and discussed.

Materials and Methods

Maintenance of organisms

Green and aposymbiotic hydra of the European strain of *Hydra viridissima* PALLAS were cultured in M solution (Muscatine and Lenhoff, 1965) at 18°C in continuous light (60 μEinsteins m^{-2} s^{-1}). Cultures were fed each Monday, Wednesday, and Friday with freshly hatched nauplii of the brine shrimp *Artemia salina* (Lenhoff and Brown, 1970). Green hydra were symbiotic with either the native strain of *Chlorella* algae (E/E hydra) or were derived from aposymbionts artificially reinfected with NC64A (E/NC hydra) or 3N813A (E/3N hydra) strains of *Chlorella* originally isolated from *Paramecium bursaria* (Muscatine *et al.*, 1967; Weis, 1978). All experimental hydra had been fed 72 h prior to use and each bore a single bud. Cultured 3N813A algae were grown as previously described (McAuley, 1986a).

Radioactive labelling of brine shrimp

Freshly hatched nauplii of *Artemia salina* were incubated at a density of 30 nauplii ml^{-1} in artificial seawater (Tropic Marin: Dr. Beiner Aquarientechnik, Wartenberg, W. Germany) containing 0.5 μCi ml^{-1} L-[4,5-^{3}H] leucine (Amersham International plc, England). After 24 h in darkness at 25°C (normal hatching conditions), *Artemia* were washed with M solution and added to dishes containing hydra (two shrimp per hydra). Determination of radioactivity in homogenates of *Artemia* showed that on average each contained 7260.7 ± 390.3 dpm (mean ± S.E. of five separate determinations). Almost 80% of the label was associated with TCA-ethanol insoluble material, suggesting that most of the leucine had been incorporated into protein.

Separation of radioactivity in algal and animal fractions of hydra

Twenty-four hours after being fed with ^{3}H-leucine labelled *Artemia*, hydra were washed in M solution, homogenized, and the volume made up to 1.5 ml. Three 100-μl aliquots of homogenate were immediately counted (total radioactivity), a further three were taken to determine total protein content, and numbers of algae were determined by counting a diluted aliquot using a hemocytometer. The algae in the rest of the homogenate were washed with 0.05% (w:v) SDS and distilled water (McAuley, 1986c), slightly modifying the technique to take account of the small volumes used. The washed algal pellet was finally resuspended in 0.45 ml distilled water. Three 100-μl aliquots were counted for radioactivity and a further 100 μl was diluted and numbers of algae determined so that dpm per SDS-washed algal cell could be calculated. Radioactivity was measured by making aliquots up to 1 ml with distilled water in a plastic scintillation vial, adding 8 ml scintillation fluid (1000 ml toluene, 500 ml Triton X-100, 6 g 2-5-diphenyloxazole), and counting using a Beckman LS1801 scintillation counter. Disintegrations per minute were calculated using the H number method.

Protein content of homogenates and SDS washed algae

Samples were freeze-thawed, extracted with an equal volume of 0.4 *M* NaOH for 1 h, and protein content determined as previously described (McAuley, 1986c).

Thin layer chromatography

Ethanolic extracts of SDS-washed algae were spotted on a TLC plate together with a small volume containing unlabelled authentic amino acids. Chromatograms were run as previously described (McAuley, 1987). Amino acids were identified by spraying with ninhydrin; spots were scraped off and suspended in scintillation fluid (1000 ml toluene, 5 g 2-5-diphenyloxazole, 0.3 g (1,4-bis[5-phenyl-2-oxazolyl]-benzene; 2,2′-p-phenylene-bis[5-phenyloxazole), and radioactivity counted.

Results

Effectiveness of SDS-washing in removal of animal contamination

To test if SDS-washing removed significantly more animal contamination from the algal pellet than simply washing with distilled water, E/E hydra were fed ^{3}H-leucine labelled *Artemia*, homogenized after 24 h, and the algae in the homogenate washed either with distilled water (controls) or 0.05% SDS. The algal pellets washed with distilled water contained almost nine times the radioactivity of those washed with SDS, while comparison of the amount of label per algal cell (as recovery of algae is lower after SDS-washing) showed that controls appeared to contain six times as much radioactivity as algae washed with SDS (Table I). To test if SDS washing removed only radioactivity associated with contaminating host material, and not radioactivity already sequestered by the algae, cultured 3N813A algae (10^7 cells per ml in 20 m*M* phosphate buffer, pH 6.3) were incubated in 0.1 m*M* ^{3}H-leucine (5 mCi per mmol) for 1 h, diluted with ice cold buffer to stop uptake, and then washed either with SDS and distilled water, or with distilled water only. Aliquots were filtered onto glass fiber disks and radioactivity was determined as previously described (McAuley, 1986a). Radioactivity per cell in algae washed with either SDS or distilled water was compared to that in algae filtered immediately at the end of the incubation period (controls). Paired *t*-tests of four replicate experiments showed no significant differ-

TABLE I

Comparison of apparent uptake of ^{3}H-label by algae isolated from hydra fed with ^{3}H-leucine labelled shrimp by conventional or SDS-washing techniques

	dpm/100 μl algal pellet	no algae × 10^{5}/ 100 μl pellet	dpm/algal cell
Wash			
Distilled water	9537.6 ± 1390.1	7.241 ± 0.895	0.01318 ± 0.00142
SDS	830.3 ± 68.6	3.716 ± 0.451	0.00223 ± 0.00032

Eighty hydra were homogenized 24 h after being fed with ^{3}H-leucine labelled *Artemia* and aliquots of the homogenate were washed with distilled water or SDS and numbers of algae and radioactivity determined as described in Materials and Methods. Figures are means ± S.E. of four independent experiments.

ence ($P > 0.10$) in radioactivity retained by controls (0.01050 ± 0.00099 dpm per cell) compared to radioactivity retained after washing with either SDS (0.00993 ± 0.00096 dpm per cell) or distilled water (0.01014 ± 0.001172 dpm per cell). Therefore, it was concluded that loss of radioactivity from algae during SDS washing was insignificant.

Further experiments determined whether all label associated with contaminating host material could be removed from the algal pellet by SDS-washing. Aposymbiotic hydra were homogenized 24 h after having been fed with ^{3}H-leucine labelled *Artemia,* and appropriate numbers of unlabelled cultured 3N813A algae were suspended in the homogenate and washed either with distilled water or with SDS. Counts showed that over 10% of radioactivity added to the algae remained after washing with distilled water, but less than 0.2% remained after washing with SDS (Table II). The results in Tables I and II suggest that if algae are washed only with distilled water, 80% of the radioactivity apparently associated with them is due to animal contamination. This is almost entirely removed by washing with SDS. A similarly high level of contamination was measured in zooxanthellae isolated from tentacles of specimens of the sea anemone *Aiptasia pulchella* which had been fed *Artemia* labelled with ^{35}S-methionine (Steen, 1986a).

TABLE II

Effect of SDS washing on removal of contaminating aposymbiotic hydra homogenate from unlabelled cultured 3N813A cells

	Experiment 1		Experiment 2	
	dpm	%	dpm	%
Label added/100 μl algae recovered	19821.9 ± 125.8	100.00	22562.2 ± 235.2	100.00
Label/100 μl algae washed with distilled water	2127.8 ± 40.7	10.74	3019.6 ± 36.8	13.38
Label/100 μl algae washed with 0.05% SDS	8.6 ± 0.5	0.04	33.7 ± 2.6	0.15

Appropriate numbers of unlabelled cultured 3N813A cells were suspended in homogenate of aposymbiotic hydra which had been fed with ^{3}H-leucine labelled *Artemia* 24 h previously. Radioactivity was determined before and after washing algal cells with distilled water or 0.05% SDS. Figures are means ± S.E. of four replicate aliquots.

TABLE III

Partition of ^{3}H-label in green hydra

	Total dpm/100 μl homogenate	dpm/algal cell	dpm/total algae in 100 μl	% label in algae
Symbiosis				
E/E	34803.3 ± 3575.9	0.003493 ± 0.00030	1191.4 ± 141.5	3.4
E/3N	30574.3 ± 2820.7	0.005592 ± 0.000564	1304.2 ± 178.7	4.3
E/NC	24263.2 ± 2694.9	0.002220 ± 0.000193	694.0 ± 53.7	2.9

Forty hydra were homogenized in 1.5 ml 24 h after having been fed ^{3}H-leucine labelled *Artemia*, and label in animal and algal fractions determined as described in Materials and Methods. Figures are means ± S.E. of four independent experiments.

Partition of ^{3}H-label supplied to green hydra by holozoic feeding

To measure movement of amino acid from host food to symbiotic algae, green hydra were fractionated (see procedure in Materials and Methods) 24 h after being fed with ^{3}H-leucine labelled *Artemia*. Transfer of ^{3}H-label to symbiotic algae occurred both in the normal (E/E) symbiosis and in artificial (E/3N and E/NC) symbioses, amounting to 3–4% of the total radioactivity retained by the hydra (Table III). In one experiment, amino acids were extracted in 80% ethanol from algae isolated from E/E hydra which had been fed ^{3}H-leucine labelled shrimp 24 h previously. Extract was co-chromatographed with authentic markers, and ninhydrin positive spots were scraped off and radioactivity determined. About 10% of identified ^{3}H-label in the ethanol-soluble pool was arginine, about 7% alanine, 3–5% each of aspartate, glutamine, and glutamate, and over 70% leucine. These results suggest that symbiotic algae were able to take up amino acids supplied to green hydra via holozoic feeding.

Further comparisons were made on the basis of specific activities per unit animal and algal protein in E/E and E/NC hydra. The total amount of protein in samples was determined directly, while the amount in algal cells was calculated from separate determinations of algal cell protein content and estimation of numbers of algae per sample. Calculations showed that in both symbioses, algal cells contained less radioactivity per unit protein than hydra tissue (Table IV). The amount of radioactivity per unit protein in E/E algae was about 24%, and in E/NC algae 18%, that per unit

TABLE IV

Partition of ^{3}H-label in green hydra according to protein content of animal and algal fractions

	Protein/algal cell (pg)	Protein in algae/ 100 μl homogenate (μg)	dpm/μg algal protein	Protein in animal/ 100 μl homogenate	dpm/μg animal protein
Symbiosis					
E/E	13.08 ± 0.14	4.44 ± 0.33	268.6 ± 23.0	31.61 ± 2.66	1109.8 ± 202.7
E/NC	10.85 ± 0.43	3.44 ± 0.33	204.6 ± 17.8	21.99 ± 2.49	1145.7 ± 229.5

Protein content of algal cells measured in three (E/NC) or four (E/E) separate determinations in 2–4 replicate samples containing 5×10^6 SDS-washed algae. Protein content of animal and algal fractions determined from measurements of total protein/100 μl homogenates (from experiments described in Table III), number of algae/100 μl and protein content of algal cells. Figures are means ± S.E. of four independent experiments.

protein of hydra tissue. This suggested that availability to the algae of amino acid derived from holozoic feeding was restricted, either because of some host mechanism directly controlling supply, or because a large proportion was immediately sequestered by host cells upon entry.

Retention of ^{3}H-label by symbiotic algae

The amount of radioactivity retained by symbiotic algae was measured for five days subsequent to feeding green hydra with ^{3}H-leucine labelled *Artemia*. At 24-h intervals, 40 hydra were homogenized, total dpm in aliquots of homogenate and per SDS-washed alga were determined, and the percentage of the total dpm found in the algae was calculated as described in Materials and Methods. Net uptake by the algae of radioactively labelled amino acid was found to continue up to 48 h after feeding (Fig. 1), but thereafter dpm per algal cell slowly declined. In contrast, the percentage of the total label retained by the symbiosis which was found in the algal population continued to increase over the period of measurement. This difference may be due to either of two reasons. First, uptake of amino acid by the algae could have been diluted by algal cell division; although algal mitosis reaches a maximum about 12 h after host feeding, it continues, although at a declining rate, for several days thereafter (McAuley, 1982). Second, increase in the proportion of label retained by algae despite a fall in dpm per algal cell may be a result of differences in the ability of algal cells and animal tissue to retain radioactively labelled amino acid. Possibly, the rate of protein catabolism was faster in host cells than in symbiotic algae during host starvation.

Discussion

Contamination of the algal pellet with host material has previously been a severe constraint on determining how radioactive metabolites are partitioned between the

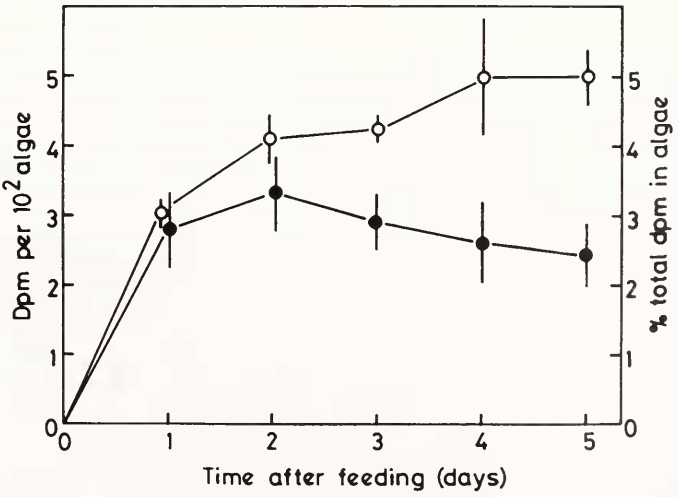

FIGURE 1. Retention of ^{3}H-label by symbiotic algae in E/E hydra fed ^{3}H-leucine labelled *Artemia*. Hydra were fed radioactive brine *Artemia* as described in Materials and Methods. At daily intervals 40 hydra were homogenized and dpm per algal cell (● —— ●) and % total ^{3}H-label retained by the symbiosis found in algal cells (○ —— ○) were calculated. Each point is the mean ± S.E. of three independent experiments.

host and its symbionts in algal/invertebrate symbioses. Although conventional washing by repeated centrifugation and resuspension in fresh medium will remove up to 90% of contaminating host material from algae isolated from green hydra (Cook, 1972; Douglas and Smith, 1983), the remaining 10% may contain several times the radioactivity actually sequestered by the algae. Contamination represents at least the same amount of protein as contained by algal cells (Douglas and Smith, 1983; McAuley, 1986a), and results described here showed that host material contained three to four times as much radioactivity per unit protein as did the algae. Therefore, unless contamination is accurately quantified or completely removed, uptake of radioactivity by symbiotic algae *in vivo* is liable to be severely overestimated.

In our experiments, SDS-washing was shown to remove from the algal pellet all but a fraction of 1% of radioactive label associated with host contamination. This enabled accurate measurement of the partition of label between host and symbiotic algae in hydra that had been fed with ^{3}H-leucine labelled *Artemia salina* nauplii. In both the natural symbiosis (E/E) and in artificial symbioses with *Paramecium* algae (E/NC, E/3N), radioactivity moved into the algae after hydra were fed with ^{3}H-leucine labelled *Artemia*, and algae in E/E hydra were found to retain significant amounts of radioactivity for at least five days after feeding.

Although the algae constitute about 15% of the total protein content of the symbiosis, only 3–4% of radioactivity retained by green hydra was found in the algae after 24 h, rising to just over 5% after 120 h. This suggests that access by the algae to amino acids derived from host digestion of food may be restricted. Since amino acids must pass through the host-derived vacuolar membrane which surrounds each alga, it is possible that the host cell can directly control the rate of amino acid supply (McAuley, 1986b). Carroll and Blanquet (1984) found that a low molecular weight factor isolated from host tissue inhibited uptake of alanine by zooxanthellae symbiotic with the jellyfish *Cassiopea xamachana*. Alternatively, differences in specific labelling of algal and animal protein may be due to competition between uptake by the algae and host utilization of amino acid for protein synthesis. That algae contain less label than expected also may be due to recycling to the host of a proportion of sequestered amino acid.

Demonstration of 'reverse translocation' suggests that maintenance of the green hydra symbiosis depends on flow of metabolites in both directions. Not only is maltose exported from the algae and used by the host (Mews, 1980; Mews and Smith, 1982), but amino acids and possibly other metabolites (McAuley, 1986a) derived from host feeding are transported into the perialgal vacuole and used by the symbiotic algae. The significance of reverse translocation is two-fold.

First, as suggested by Douglas and Smith (1983), supply of metabolites to the algae may impose a net 'cost' upon the host in certain circumstances. When hydra are grown in continuous darkness, where maltose release is reduced and the algae must depend upon host-derived metabolites for growth, those with a population of algae grow more slowly than aposymbionts artificially rid of their algae (Douglas and Smith, 1983). A similar net cost has been found in the sea anemone *Aiptasia pulchella*, whose symbiotic zooxanthellae sequester radioactivity when hosts are given ^{35}S-methionine labelled food (Steen 1986a). Symbiotic anemones starved in darkness showed a significant decline in the adenylate ratio of ATP: (ATP + ADP) compared to aposymbionts, but in light the adenylate ratio declined at the same rate in starved symbiotic and starved aposymbiotic anemones (Steen, 1986b).

Second, there is now persuasive evidence that uptake of ammonium by algae in symbiosis with green hydra is prevented by low perialgal vacuolar pH and high levels of host glutamine synthetase (Rees, 1986). Furthermore, measurements of amino

acid pool size and of uptake and metabolism of amino acids indicate that symbiotic algae may be nitrogen-limited (McAuley, 1986b, 1987). Experiments described here, demonstrating movement of ^{3}H-leucine from host food to symbiotic algae, support the suggestion that in green hydra amino acids may form the principal supply of nitrogen in symbiosis (McAuley, 1986b, 1987; Rees, 1986). This differs from symbioses between marine invertebrates and zooxanthellae, in which nitrogen is supplied to the symbionts as ammonium (Muscatine, 1980; Wilkerson and Muscatine, 1984; Summons *et al.*, 1986; Anderson and Burris, 1987). These experiments also suggested that *in vivo* uptake of ^{3}H-leucine by symbiotic algae may be restricted. Host control of supply of nitrogen, or of one or more specific amino acids, may be the mechanism whereby cell growth of symbiotic algae is reduced, and cell division is inhibited except at host cell division (McAuley, 1981, 1985, 1986d).

ACKNOWLEDGMENTS

I thank Professor Sir David Smith, Biological Secretary, Royal Society for suggesting improvements to a draft of this paper. This work was supported by the Science and Engineering Research Council.

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